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Contact with water functions as a Zeitgeber for the circatidal rhythm in the mangrove cricket *Apteronemobius asahinai*

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The mangrove cricket *Apteronemobius asahinai* shows a circatidal rhythm in its locomotor activity, and this rhythm was shown to be entrained to artificial tidal cycles in the laboratory. To examine the Zeitgeber for this rhythm, in the present study, crickets were fixed with insect pins to prevent their body locomotion and a water stimulus was given to them by soaking in water while recording their locomotor activities. A single water stimulus delayed the phase when given in the middle subjective low tide and advanced the phase when given in the later subjective low tide, whereas it had only a slight effect in the subjective high tide. We conclude that contact with water functions as a Zeitgeber for the circatidal rhythm.

Keywords: circatidal rhythm, entrainment, locomotor activity, mangrove cricket, phase response curve, Zeitgeber

Introduction

Circatidal rhythms have been reported in some organisms, e.g., the fiddler crabs *Uca pugnax* and *U. pugilator*, the amphipod *Synchelidium*, the saltmarsh collembolan *Anurida maritima*, the cumacean crustacean *Dimorphostylis asiatica*, and the limpet *Helcion pectunculus*, and most of them live in the intertidal zone (Bennett et al. 1957; Barnwell 1966; Enright 1963; Foster and Moreton 1981; Akiyama 1995; Grey & Hodgson 1999). The mangrove cricket *Apteronemobius asahinai*, inhabits the floor of mangrove forests, where tidal submergence is repeated with a period of approximately 12.4 h. The locomotor activity of this species shows a circatidal rhythm with a free-running period of approximately 12.6 h to adapt to the ebb and flow of tides (Satoh et al. 2008). This rhythm has been shown to be produced by a circatidal clock different from the circadian clock, as in *Carcinus maenas* (Satoh et al. 2009; Takekata et al. 2012, 2014; Naylor 1996). However, the anatomical location of the circatidal clock in *A. asahinai* is still unclear (Takekata et al. 2014).

Because circatidal rhythms are mechanisms for adaptation to tidal cycles, these rhythms should entrain to tidal cycles under natural conditions. Environmental cues for entrainment of biological rhythms are called Zeitgebers. It is necessary to identify the Zeitgeber in order to clarify the anatomical location of the receptor organ for entrainment and the input pathway to the clock producing the rhythm. Various kinds of Zeitgebers for circatidal rhythms have been reported: e.g., salinity, hydrostatic pressure, water temperature, and mechanical stimulation, including wave action, water turbulence and mechanical agitation (Taylor & aylor 1977; Forward et al. 1986; Morgan 1965; Reid & Naylor 1990; Akiyama 2004; Naylor 1963; Holmström & Morgan 1983; Enright 1965; Jones & Naylor 1970; Ehlinger & Tankersley 2006). In *A. asahinai*, Satoh et al. (2009) showed that, in the laboratory, the circatidal rhythm is entrained to artificial tides produced by a special apparatus. This apparatus automatically inundates the recording chambers with a period of 12.4 h for 30 min during the measurement of the locomotor activity. However, it is still unclear whether contact of a cricket's body with water or exogenous periodic disturbance causing body locomotion acts as an actual Zeitgeber. The discrimination between these two possibilities is important in order to clarify the neural mechanism of the circatidal rhythm because the input pathways to the clock producing the rhythm must be different between them. The purpose of the present study was to clarify whether the circatidal rhythm can be entrained only by contact with water in *A. asahinai*. We examined the phase shifts produced by water submergence after a cricket body is fixed to prevent its body locomotion.

Materials and methods

Insects

Adult males of *A. asahinai* were collected from mangrove swamps in Ginoza, Okinawa Prefecture, Japan (26°30'N, 127°59'E) between August 2015 and October 2016, and used for the locomotor activity recording from the day of collection.

Recording of locomotor activity

Activity was recorded according to Takekata et al. (2012). Crickets were housed individually in plastic petri dishes (30 mm in diameter), in which sufficient food was fixed with an adhesive at one end and the wetted cotton passed from the water source was set at the other end. The food used for recording was made from insect pellets (Oriental Yeast, Tokyo, Japan), agar and propionic acid. The petri dishes were placed in an incubator maintained at $25.0 \pm 1.0^{\circ}\text{C}$ under constant dim red light at approximately 14.6 mW/m^2 with an LED (HLMP-EH22, Avago Technologies, San Jose, California, USA). Infrared beams (EE-SPW321, Omron, Kyoto, Japan) were installed across the petri dishes and the number of interruptions of the beam was counted at 6 min intervals.

Water-stimulus procedures

A petri dish containing a cricket was taken out of the incubator and placed in ice for 3 min for cold anesthesia. The anesthetized cricket was immediately fixed by insect pins on a wax board (Figure 1). The fixed cricket was soaked in water at a depth of 5 mm for 30 min (water stimulus). In the control group, crickets were anesthetized, fixed, and kept without water stimulus for 30 min. After the treatment, the crickets were returned to the petri dishes and the activity recording was resumed.

Analysis of rhythms

Circatidal rhythmicity was determined by using a chi-square periodogram (Sokolove & Bushell 1978; Takekata et al. 2012). The phases of the circatidal rhythm before and after the stimulus were detected with the eye-fit method by three persons independently. The average of the slopes and intercepts determined by the three persons were used for the calculation of the phase. The phase shifts were calculated as the difference between these phases at the cycle next to the one when the stimulus was given. A phase response curve (PRC), which explains the relation of the phase shift and the phase when a stimulus was given, was calculated using the following formula based on Minors et al. (1991):

$$PRC(p) = \frac{\sum_{i=1}^n y_i I_A(x_i)}{\sum_{i=1}^n I_A(x_i)}$$

Where $PRC(p)$ is the value of the estimated phase shift at p , the phase when the stimulus was given ($0 < p \leq 360$); i is an individual number ($i = 1, 2, 3, \dots, n$); n is the number of crickets used (22 and 31 for the experimental and control groups, respectively); y_i is the value of the observed phase shift at x_i , the phase when the stimulus was given; $I_A(x_i)$ is an indicator function, which returns 1 if x_i is in a set A and returns 0 if not; D is an integer (35 in the present study); When $D \leq p \leq 360 - D$, A is a set, $\{x \mid p - D \leq x \leq p + D\}$, when $0 < p \leq D$, A is a union of sets which are $\{x \mid 0 < x \leq p + D\}$ and $\{x \mid p - D + 360 \leq x \leq 360\}$ and when $360 - D \leq p \leq 360$, A is a union of sets which are $\{x \mid p - D \leq x \leq 360\}$ and $\{x \mid 0 < x \leq p + D - 360\}$. This method means that an estimated phase shift at a phase when a stimulus was given is the average of the observed values of phase shifts within the range between the designated phase $\pm D$. By this method, averages of phase shifts can be continuously derived.

The uniformity of phases was examined by the Rayleigh test with the “circular” package, Version 1.1.3 in R Version 3.3.0 (Lund and Agostinelli, 2013; R Development Core Team 2013).

Results

Locomotor activity was recorded in a total of 80 crickets. Two of them showed no obvious circatidal rhythm from the start of the observation, and 1 and 2 crickets lost the rhythm by the treatment in the experimental and control groups, respectively. Moreover, 15 and 7 crickets died within 5 days after the treatment in the experimental and control groups, respectively. The remaining 53 crickets were used for the analysis.

There were no significant differences between the free-running periods of the circatidal rhythm before and after the treatment in either the experimental or the control groups (Table 1). Thus, not only a water stimulus but also cold anesthesia and subsequent fixation of the body did not affect the period of the circatidal rhythm. A single water stimulus produced a phase-dependent phase shift. A water stimulus delayed the phase when given in the middle subjective low tide (Figure 2A) and advanced the phase when given in the later subjective low tide (Figure 2B) whereas it had only a slight effect when given in the subjective high tide (Figure 2C). In the control group, however, there was no simple relationship between the phase of the treatment and the phase shift produced, and Figure 2D shows an example with no phase shift when the treatment was given at the subjective low tide.

We constructed PRCs from the phase shifts in the experimental and control groups. A single water stimulus produced a phase shift depending on the phase at which the stimulus was given (Figure 3A), although in the control group the phase was not prominently shifted on average irrespective of the phase at which the treatment was

given (Figure 3B). Based on comparison of the two PRCs, we suggest that the water stimulus functions as a Zeitgeber for the circatidal rhythm.

To further confirm that the contact with water is a Zeitgeber for the circatidal rhythm, we examined whether the water stimulus shifted the phases into a certain range. If the contact with water is a Zeitgeber, the phases were expected to move to the subjective high tide after the water stimulus was given, as shown by Satoh et al. (2009). The distribution of phases after the water stimulus was significantly different from the uniform distribution, although that before the water stimulus was not (Figure 4AB). In contrast, neither the distribution of phases before or after the treatment in the control group was significantly different from the uniform distribution (Figure 4CD). Adult males of *A. asahinai* were collected from mangrove swamps in Ginoza, Okinawa Prefecture, Japan (26°30'N, 127°59'E) between August 2015 and October 2016, and used for the locomotor activity recording from the day of collection.

Discussion

Satoh et al. (2009) showed that the circatidal rhythm of *A. asahinai* is entrained to artificial tides in the laboratory, although it was still unclear whether the Zeitgeber is actually contact of a cricket's body with water or rather a disturbance of the cricket's behavior. In fact, exogenous disturbance plays a role as a Zeitgeber for circatidal rhythms in the cirolanid isopod *Exciorolana chiltoni*, the American horseshoe crab, *Limulus polyphemus*, and the sand beach isopod *Eurydice pulchra* (Enright 1965; Ehlinger & Tankersley 2006; Jones & Naylor 1970). The present results, however, clearly showed that contact with water functions as a Zeitgeber for the circatidal rhythm in *A. asahinai* but disturbance of behavior does not.

In *A. asahinai*, Takekata et al. (2014) showed that the circatidal rhythm was maintained and the circadian rhythm disappeared after bilateral removal of the optic

lobes, in which the circadian clock resides, and concluded that the circatidal clock is anatomically different from the circadian clock. The present results provide a clue to clarify the neural mechanism of the circatidal clock in *A. asahinai*. Because contact with water functions as a Zeitgeber for the circatidal rhythm, water receptors should be neutrally connected to the clock, like the compound eyes for the circadian clock in the optic lobe (Nishiitsutsuji-Uwo & Pittendrigh 1968; Tomioka & Chiba 1984). Kanou et al. (2007) described water receptors on legs in the two-spotted cricket, *Gryllus bimaculatus*, and suggested that these receptors function for initiation of swimming. Here we point out the possibility that water receptors on the legs receive a water stimulus for entrainment of the circatidal rhythm in *A. asahinai*, and plan to examine this hypothesis to clarify the neural pathway to the circatidal clock.

Disclosure statements

No potential conflict of interest was reported by the authors.

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Table 1. Free-running periods of the circatidal rhythm in *Apteronemobius asahinai* before and after a water stimulus.

Treatment group	<i>n</i>	Free running period	
		(mean \pm S.D., h)	
		Before treatment	After treatment
Water stimulus	22	12.62 \pm 0.24	12.63 \pm 0.26
Control	31	12.52 \pm 0.17	12.58 \pm 0.23

There was no significant difference between the periods before and after the treatment in either group by the paired *t* test ($P > 0.05$).

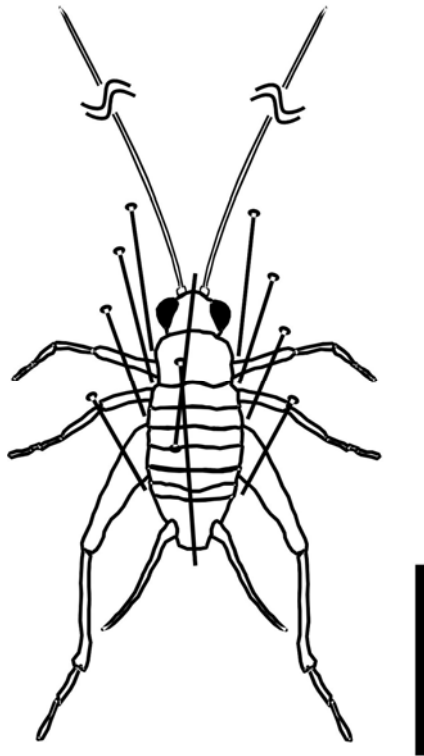
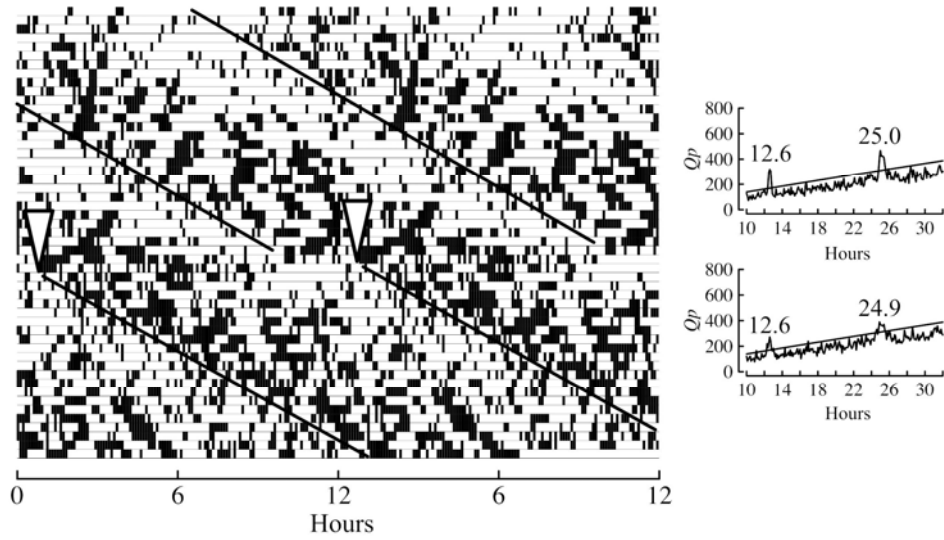
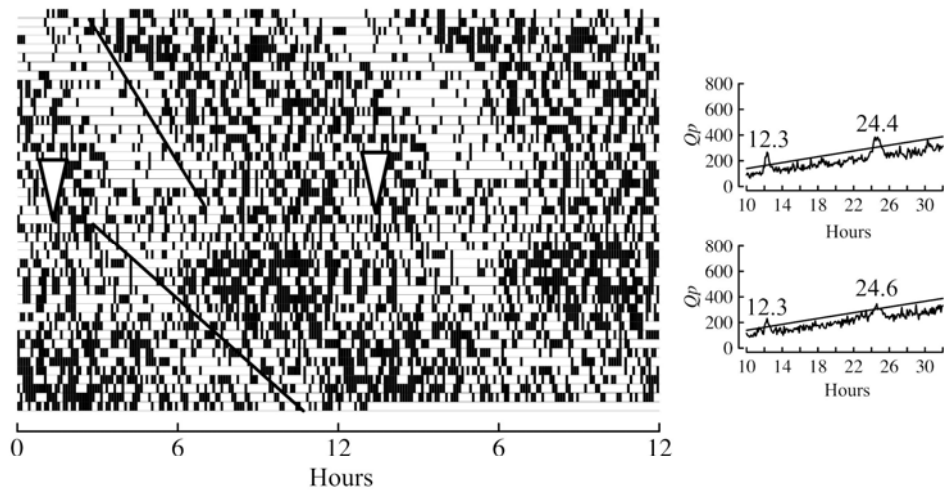


Figure 1. Schematic diagram of *Apteronomobius asahinai*, fixed by 10 insect pins on a wax board. Eight pins were inserted into the regions anterior to fore-legs, between fore- and mid-legs, between mid- and hind-legs, and posterior to hind-legs on the left and right sides of the cricket. Two other pins were put at the anterior and posterior ends of the cricket. Scale bar, 5 mm.

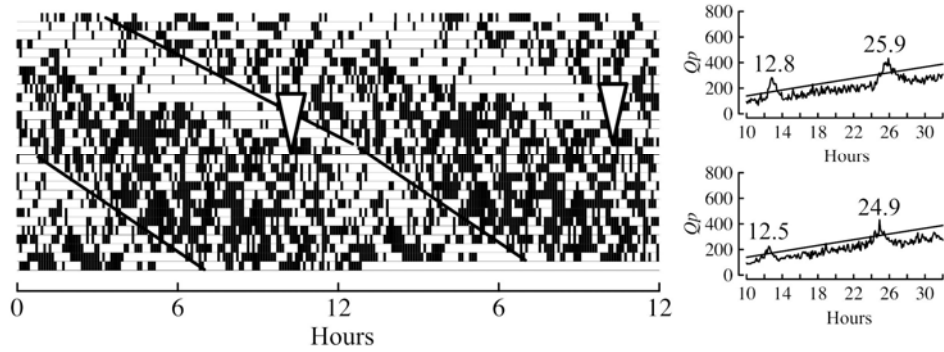
A



B



C



D

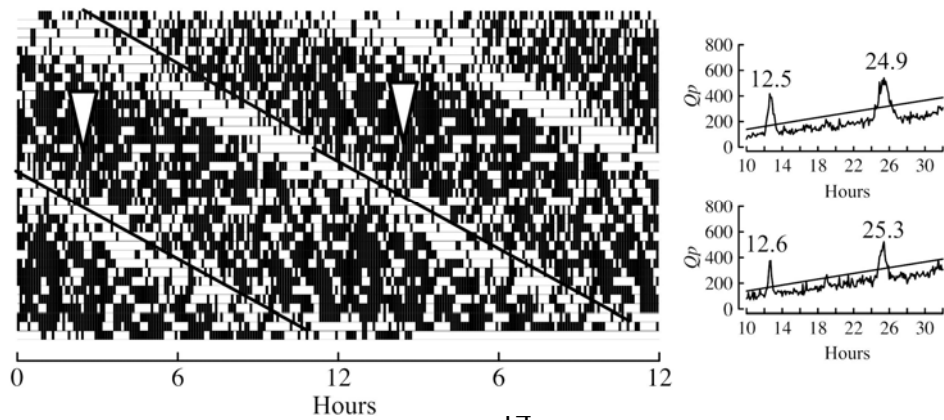


Figure 2. Representative locomotor activity rhythms of adult males of *Apteronemobius asahinai*. A water stimulus was given in the middle subjective low tide (A), in the later subjective low tide (B) and in the subjective high tide (C). In the control group, crickets were anesthetized and fixed without water stimulus in the subjective low tide (D). (i) The activities presented by double-plotted actograms (12h \times 2). Inverted triangles on the actogram show the time of the stimulus. The oblique lines show the estimated beginnings of active phases. The chi-square periodograms of activities before (ii) and after (iii) the stimulus are also shown. The oblique line in the periodogram corresponds to the significance level of $\alpha = 0.005$ of each chi-square test. The peak values above the oblique line were determined to be significant.

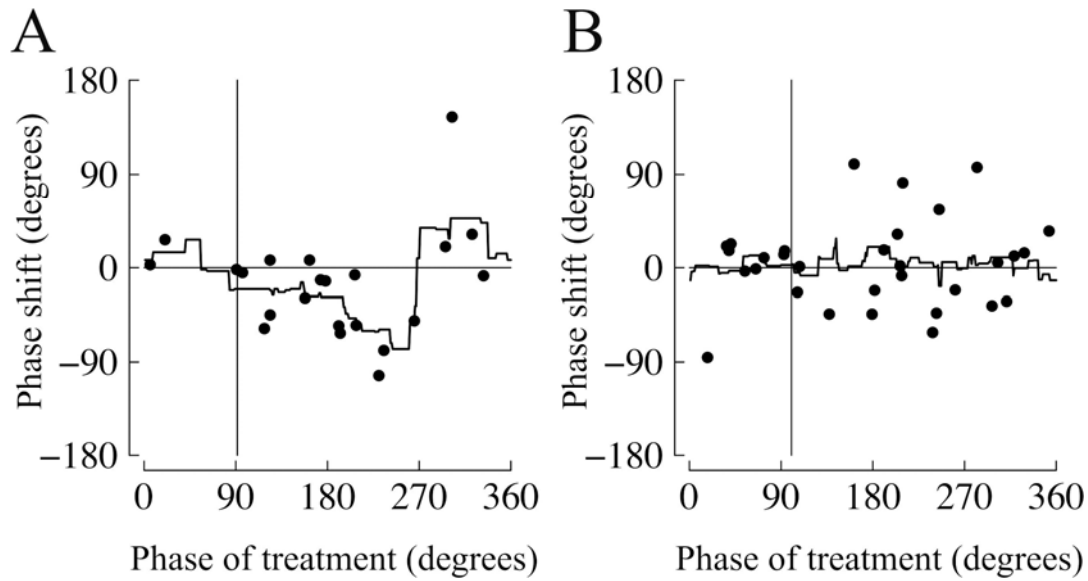


Figure 3. Phase response curves to a single water stimulus in the circatidal rhythm of *Apteronomobius asahinai*. In the experimental group, a single water stimulus was given at various phases of the free-running circatidal rhythm (A). In the control group, crickets were anesthetized and fixed without a water stimulus (B). Phases are shown in terms of angle. The vertical lines indicate the average end of the subjective high tide. The curves indicate the continuous averages of the observed phase shifts. See Materials and Methods for details.

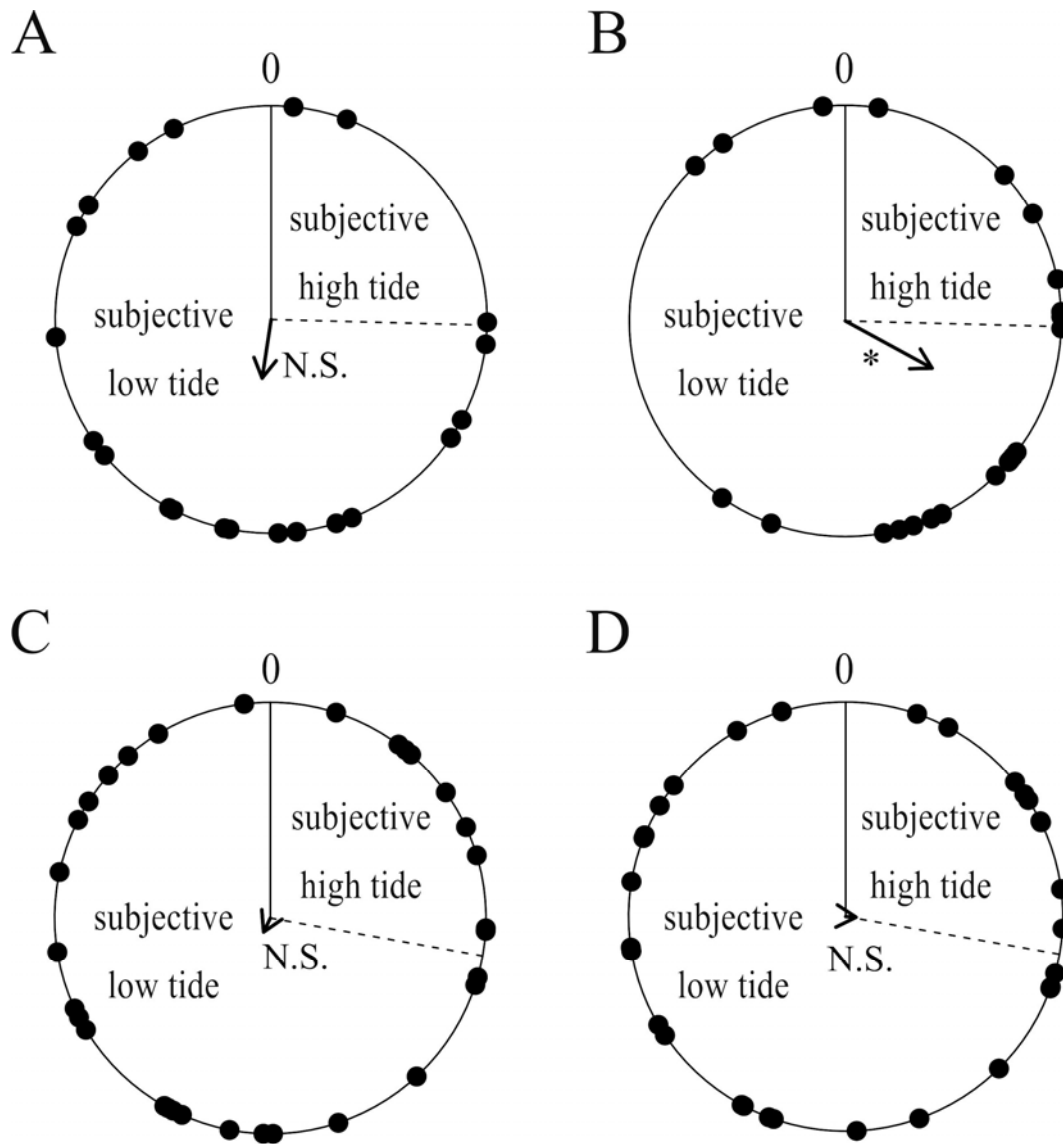


Figure 4. Circular statistics of the phases before and after a water stimulus in the circatidal rhythm in *Apteronomobius asahinai*. In the experimental group, a single water stimulus was given at various phases of the free-running circatidal rhythm (A, B), whereas in the control group, crickets were anesthetized and fixed without a water stimulus (C, D). Closed circles show the phases before and after the treatment in individual crickets. The horizontal solid lines represent the beginning of the subjective high tide (0°), and the broken lines indicate the average end of the subjective high tide. The direction of vectors (arrows) indicates the average angle, and its length indicates

the dispersion of the angles. * $P < 0.05$; N.S. $P > 0.05$ by the Rayleigh test examining uniformity of the distribution. Type your caption here. Obtain permission and include the acknowledgement required by the copyright holder if a figure is being reproduced from another source.